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LITTON BIONETICS INC

**Document Title**

MUTAGENICITY EVALUATION OF 10558-10B IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY (FINAL REPORT) WITH COVER LETTER DATED 011691

**Chemical Category**

TOULENE DIISOCYANATE (26471-62-5)

CONTAINS NO CBI

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Attn: Health and Safety Reporting Rule (Notification/Reporting)

Please find attached 8(d) health and safety reports for mixtures processed containing toluene diisocyanate (CAS #26471-62-5), 4,4-Diphenylmethane diisocyanate (CAS #101-68-8) and 1,6-Diisocyanatohexane (CAS #822-06-0). Grace is submitting these reports for late filing since their submittal may have been subject to the isocyanates 10-year call-in of June 1, 1987.

We have reason to believe that some of these reports may have previously been submitted to EPA as attachments to PMN submissions. However, Grace is filing them as a precautionary measure to insure EPA's receipt.

These reports are being submitted for:

W. R. Grace & Co.-Conn.
Washington Research Center
7379 Route 32
Columbia, MD 21044

Sincerely,

J. W. Raksis

A:\JR91-013/lw

Attachments - 20

86910000638

CONTAINS NO CBI

Genetics Assay #3095

Toluene Diisocyanate
26471-62-5
1,6-Diisocyanatohexane
822-06-0

# MUTAGENICITY EVALUATION OF

10558-10B

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

W. R. GRACE and COMPANY
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COLUMBIA, MARYLAND 21044

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
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LBI PROJECT NO. [illegible]

NOVEMBER, 1978



Litton BIONETICS

## PREFACE

This report contains a summary of the data compiled during the evaluation of the test material. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IV and provides sponsor and compound identification information, type of assay and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V identifies the tables and/or figures containing the data used by the Study Director in interpreting the test results (item VI).

The second part of the report, entitled PROTOCOL, describes, in detail, the materials and procedures employed in conducting the evalution. This part of the report also contains evaluation criteria, standard operating procedures and any appendices. These are included to acquaint the sponsor with the methods used to develop and analyze the test results. All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology. Copies of raw data will be supplied to the sponsor upon request.

I. SPONSOR W.R. GRACE

II. MATERIAL

A. Identification: 10558-10B

B. Date Received: April 19, 1978

C. Physical Description: Viscous yellow liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. PROTOCOL NO.: DMT-106

V. RESULTS

The data presented in Table 1 show the concentrations of the test compound employed, number of mutant clones obtained, surviving populations after the expression period, and calculated mutation frequencies. All calculations are performed by computer program.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound, 10558-10B, was soluble in DMSO at a concentration of 50 µl/ml. It was tested in the mutation assay at applied doses ranging from 0.975 nl/ml to 500 nl/ml for the nonactivation and the activation tests. This dose range was chosen on the basis of a preliminary cytotoxicity test which indicated that doses higher than 20 nl/ml without activation were highly toxic to mouse lymphoma cells. In the mutation assay, doses higher than 15.6 nl/ml without activation and 31.25 nl/ml with activation destroyed nearly all the cells within 24 hours of treatment and therefore were deleted from further testing. Low dose levels were eliminated on the basis of insufficient cytotoxicity.

The doses chosen for completion of the mutagenesis assay (see Table 1) were within the range of cytotoxicities where any mutagenic activity is normally observed. After the cells were cloned for mutant selection, the percent relative growth in the treated cultures ranged from 149% to 31% without activation and from 176% to 29% with activation. The results of the mutation assay are presented in Table 1.

With and without activation, the mutant frequencies in the cultures treated with the test compound were all comparable to the solvent and untreated negative control values. Even at the relatively toxic doses of 15.6 nl/ml without activation and 31.3 nl/ml with activation, no increase in mutant frequency was observed.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The validity of the mutagenesis assay can be assessed by the results obtained for the positive and negative controls. The cloning efficiencies for the solvent and untreated negative controls varied from an average of 85% without activation to 60% with activation, which demonstrates good culturing conditions for the assay. The negative (solvent and untreated) control mutant frequencies are all within the normal range for nonactivation and activation tests, and the positive control compounds yielded frequencies in the normal range that are greatly in excess of the negative control values (background).

The test compound, 10558-10B, did not induce an increase in mutations at the TK locus in L5178Y mouse lymphoma cells at doses of 0.975 to 15.6 nl/ml without activation and at 1.95 to 31.3 nl/ml with microsomal activation.

Therefore the test compound is considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

Dates of initiation and completion of the study:
June 1, 1978 to November 20, 1978.

Submitted by:

Study Director

Brian C. Myhr 11-27-78

Brian Myhr, Ph.D. Date
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick 11/27/78

David J. Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

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4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: 10558-108
B. LBI CODE #: 3095
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 11/06/78

| TEST | S-9 SOURCE | S-9 TISSUE | DAILY COUNTS (CELLS/ML X 10E5) 1 | 2 | 3 | RELATIVE SUSPENSION GROWTH (% OF CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH* | MUTANT FREQUENCY** X 10E-6 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **NONACTIVATION** | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 10.6 | 11.8 | | 100.0 | 27.0 | 179.0 | 100.0 | 100.0 | 15.1 |
| SOLVENT CONTROL | --- | --- | 11.6 | 12.6 | | 100.0 | 34.0 | 195.0 | 100.0 | 100.0 | 17.4 |
| NEGATIVE CONTROL | --- | --- | 15.0 | 8.0 | | 88.6 | 14.0 | 390.0† | 208.6 | 184.8 | 3.6 |
| EMS .5 UL/ML | --- | --- | 6.0 | 8.8 | | 39.0 | 617.0 | 110.0 | 58.8 | 22.9 | 560.9 |
| TEST COMPOUND | | | | | | | | | | | |
| 0.975 NL/ML | --- | --- | 9.0 | 10.6 | | 70.4 | 33.0 | 259.0 | 138.5 | 97.6 | 12.7 |
| 1.950 NL/ML | --- | --- | 10.4 | 16.8 | | 129.0 | 24.0 | 216.0 | 115.5 | 149.0 | 11.1 |
| 3.900 NL/ML | --- | --- | 9.0 | 16.4 | | 109.0 | 25.0 | 95.0 | 50.8 | 55.4 | 26.3 |
| 7.800 NL/ML | --- | --- | 5.0 | 14.0 | | 51.7 | 28.0 | 229.0 | 122.5 | 63.3 | 12.2 |
| 15.600 NL/ML | --- | --- | 4.2 | 9.4 | | 29.2 | 43.0 | 201.0 | 107.5 | 31.3 | 21.4 |
| **ACTIVATION** | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 11.4 | 8.0 | | 100.0 | 30.0 | 179.0 | 100.0 | 100.0 | 16.8 |
| SOLVENT CONTROL | RAT | LIVER | 11.8 | 11.0 | | 100.0 | 29.0 | 216.0 | 100.0 | 100.0 | 13.4 |
| NEGATIVE CONTROL | RAT | LIVER | 18.0 | 11.2 | | 182.9 | 31.0 | 144.0 | 72.9 | 133.4 | 21.5 |
| DMN .3 UL/ML | RAT | LIVER | 8.6 | 6.8 | | 53.1 | 209.0 | 76.0 | 38.5 | 20.4 | 275.0 |
| TEST COMPOUND | | | | | | | | | | | |
| 1.950 NL/ML | RAT | LIVER | 10.4 | 10.8 | | 101.9 | 33.0† | 341.0 | 172.7 | 176.0 | 9.7 |
| 3.900 NL/ML | RAT | LIVER | 6.8 | 13.4 | | 82.7 | 35.0 | 267.0 | 135.2 | 111.8 | 13.1 |
| 7.800 NL/ML | RAT | LIVER | 8.4 | 11.6 | | 88.4 | 41.0 | 192.0 | 97.2 | 86.0 | 21.4 |
| 15.600 NL/ML | RAT | LIVER | 5.4 | 12.2 | | 33.2 | 47.0 | 235.0 | 119.0 | 39.5 | 20.0 |
| 31.300 NL/ML | RAT | LIVER | 3.4 | 7.4 | | 20.1 | 35.0 | 288.0 | 145.8 | 29.4 | 12.2 |

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4. THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) x 10E-4. THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
† = ONE PLATE CONTAMINATED: VALUE BASED ON REMAINING TWO PLATES.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for specific locus forward mutation induction in the L5178Y thymidine kinase (TK) mouse lymphoma cell assay.

2. MATERIALS

A. Indicator Cells

The cells used in this study were derived from Fischer mouse lymphoma cell line L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BrdU) sensitive. Scoring for mutation was based on selecting cells that had undergone forward mutation from a TK +/- to a TK -/- genotype by cloning them in soft agar with BrdU.

B. Media

The cells were maintained in Fischer's medium for leukemic cells of mice with 10% horse serum and sodium pyruvate. Cloning medium consisted of Fischer's medium with 10% horse serum, sodium pyruvate, and 0.35% Noble agar. Selection medium was made from cloning medium by the addition of 5 ml BrdU to 100 ml cloning medium.

C. Control Compounds

1. Negative Control

The solvent in which the test compound was prepared was used as a solvent (or vehicle) control and is designated as solvent control in the data table. The actual solvent is listed in Table 1 of Section V Results. A negative control consisting of media only was used to assess the base-line viability of the cells.

2. Positive Control

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and was used as a positive control for the nonactivation studies at a final concentration of 0.5 µl/ml.

Dimethylnitrosamine (DMN), which induces mutation by base-pair substitution and requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for the activation studies at a final concentration of 0.3 µl/ml.

## 3. EXPERIMENTAL DESIGN

### A. Toxicity

Ths solubility, toxicity, and doses for all chemicals were determined prior to screening. The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of cells induced by a 4 hour exposure to the chemical followed by a 24 hour expression period in growth medium. A minimum of four concentrations was selected from the concentration range by using as the highest dose, a level that showed a reduction in growth potential. At least three lower doses, including levels which were below the toxic range were added. Those compounds that were relatively nontoxic to cells growing in suspension were tested at concentrations of up to 10 mg/ml when solubility permitted. Toxicity produced by chemical treatment was monitored during the experiment.

### B. Assays

#### 1. Nonactivation Assay

The procedure used is a modification of that reported by Clive and Spector (1975). Prior to each treatment, cells were cleansed of spontaneous TK -/- by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits the survival of only those cells that produce TK, and can therefore, utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in growth medium at the predetermined doses for 4 hours at 37°C on a rocker. The treated cells were washed, fed, and allowed to express in growth medium for 2 days. At the end of this expression period, TK -/- mutants were detected by cloning the cells in the selection medium for 10 days. Surviving cell populations were determined by plating diluted aliquots in nonselective growth medium.

#### 2. Activation Assay

The activation assay differed from the nonactivation assay in the following manner only: The liver was pretested for toxicity to the cells and the appropriate volume (generally ranging from 1.0 ml to 0.5 ml) was added to 1 ml of the cofactors. This activation mixture was added to the required volume (generally, 8.0 to 8.5 ml) of medium containing the desired number of cleansed cells.

3. EXPERIMENTAL DESIGN (Continued)

C. Preparation of 9,000 x g Supernatant

Male, random bred rats (Fischer 344) pretreated with Aroclor 1254 were killed by cranial blow, decapitated, and bled. The liver was immediately dissected from the animal using aseptic technique and placed in ice-cold 0.25 M sucrose buffered with sodium phosphate at pH 7.4. When an adequate number of livers had been collected, they were washed twice with fresh buffered sucrose and completely homogenized. The homogenate was centrifuged for 20 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from this centrifuged sample was retained and frozen at -80°C until used in the activation system. This microsome preparation was added to culture medium along with the appropriate cofactors in the concentrations described below:

| Component | Final Concentration/ml |
|---|---|
| NADP (sodium salt) | 2.4 mg |
| Isocitric acid | 4.5 mg |

D. Screening

A mutation index was derived by dividing the number of clones formed in the BrdU containing selection medium by the number found in the same medium without BrdU. The ratio was then compared to that obtained from other dose levels and from positive and negative controls. Colonies were counted on an electronic colony counter that resolves all colonies greater than 200 microns in diameter.

4. EVALUATION CRITERIA

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the Mouse Lymphoma Forward Mutation Assay. These criteria are derived from a historical data base and are helpful in maintaining uniformity in evaluations from material to material, and run to run. While these criteria are reasonably objective, a certain amount of flexibility may be required in making the final evaluation since absolute criteria may not be applicable to all biological data.

4. EVALUATION CRITERIA (Continued)

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 5 dose levels employed.
- The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

STANDARD OPERATING PROCEDURE

All data will be entered in ink (no pencil).

All changes or corrections in entries will be made with a single line through the change, and an explanation for the change must be written.

All calculations (weights, dilutions, dose calculations, etc.) will be shown on data records.

All data entries will be dated and initialed.

All laboratory perations will be written out in standard protocol manuals. These manuals will be present in each laboratory area.

Deviations from any established protocol will be described and justified.

Data will be stored in bound form (notebooks or binders). These bound data books will be reviewed by the appropriate Section Heads.

Chemicals submitted for testing will have data of receipt and initials of entering person.

Lot numbers for all reference mutagens, solvent, or other materials used in assays will be recorded.

Animal orders, receipts, and identification will be recorded and maintained such that each animal can be traced to the supplier and shipment. All animals on study will be properly identified.

A copy of the final report plus all raw data and support documents will be permanently stored in the archival system of Litton Bionetics, Inc.

Current curricula vitae and job descriptions will be maintained on all personnel involved in the study.

## REFERENCES

Clive, D. and Spector, J.F.S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31, 17-29.

## CERTIFICATE OF AUTHENTICITY

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